DEVELOPMENT OF A LC-MS/MS METHOD TO DETECT AND QUANTIFY PYRIDOSTIGMINE IN PLASMA

By

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Bachelor of Science in Chemistry

Harding University

Searcy, AR

2015

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
May, 2017

DEVELOPMENT OF A LC-MS/MS METHOD TO

DETECT AND QUANTIFY PYRIDOSTIGMINE

IN PLASMA

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ACKNOWLEDGEMENTS

I would like to thank my advisor Dr. Wagner for assisting me throughout my time here at OSU-CHS. I would also like to thank Dr. Maxwell for providing me with the opportunity to work on this project. Next, I want to thank the OSU-FTTL staff including Dr. Veitenheimer and Minh Ngo for mentoring me throughout this project. Finally, I want to give a huge thank you to my family and friends that supported me these last two years. I would not have been as successful without your love and support.

Name: MAEGEN SLOAN

Date of Degree: MAY, 2017

Title of Study: DEVELOPMENT OF A LC-MS/MS METHOD TO DETECT AND QUANTIFY PYRIDOSTIGMINE IN PLASMA

Major Field: FORENSIC SCIENCES

Abstract:

Pyridostigmine is a reversible acetylcholinesterase inhibitor that has been used to help treat myasthenia gravis and reduce nerve agent effects. By reversibly binding to acetylcholinesterase, Pyridostigmine causes elevation of acetylcholine, which is beneficial in disease states where increased contractility is needed. The present study relates to the investigation of Pyridostigmine to treat cardiac patients. A sensitive and specific liquid chromatography tandem mass spectrometry (LC-MS/MS) method was developed to detect Pyridostigmine in plasma samples from study participants dosed with this drug. The method was validated according to Scientific Working Group in Forensic Toxicology (SWGTOX) guidelines and used to quantify the amount of Pyridostigmine in the patient plasma samples. Solid phase extraction (SPE) was used to extract the drug from the plasma matrix. The SPE and LC-MS/MS method successfully quantified Pyridostigmine in the study participants, facilitating the development of a dosing model by research partners.

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CHAPTER I

INTRODUCTION

Recently, the Oklahoma State University Center for Veterinary Health Sciences needed a method to quantitate Pyridostigmine (PYR) in the plasma of heart patients in a clinical trial they were supporting. In response, a method was developed that utilized solid phase extraction (SPE) followed by liquid chromatography tandem mass spectrometry (LC-MS/MS). Following development, this method was validated as being sensitive and selective for PYR in human plasma. This method provides the means to quantify PYR, which is necessary to develop models to determine proper dosing for patients treated with this drug.

Previous literature shows that PYR induces cholinergic stimulation, which in turn increases heart rate,[1] reduces ventricular arrhythmias,[2] and blunts the cardiac responses to mental stress.[3] These studies provide cardiac test results that indicate PYR is improving cardiac function. Literature also exists that provides methods to detect PYR in rat urine and guinea pig plasma, but not in humans using LC-MS/MS.[4,5] This literature indicates that PYR can help with cardiac function and that it has the potential to be accurately measured in human biological specimens.

This method was necessary to support a dosing study for PYR. The studies providing methods to detect PYR only do so for animal specimen or human specimen using only ultraviolet detection techniques. Also, no study exists that provides an LC-MS/MS method to detect and quantify PYR in human plasma.

CHAPTER II

REVIEW OF LITERATURE

2.1 Introduction

The topic for this research project is the development and validation of a solid phase extraction (SPE) and liquid chromatography tandem mass spectroscopy (LC-MS/MS) method to detect and quantify Pyridostigmine (PYR) in plasma samples. Patients involved in the clinical trial were administered PYR in an attempt to help improve cardiac function. Then, after the patients had completed heart stimulating physical activity, blood samples were taken during specific time increments. Development of a method to detect the amount of PYR in the bloodstream following controlled administration can help provide information about the pharmacokinetics and pharmacodynamics of PYR.

2.2 Background

PYR is a complex drug that has been used for many treatments. Its function in the body is as an acetylcholinesterase (AChE) inhibitor. As an AChE inhibitor, PYR has been used to help treat the symptoms caused by myasthenia gravis. This drug has also been used to help protect against nerve agent effects. Most recently, PYR has been used to try to help improve cardiac function.

2.2.1 PYR as an AChE Inhibitor

As mentioned, PYR is an AChE inhibitor. Cholinesterase inhibitors are compounds that keep the enzyme AChE from functioning. During neurotransmission, acetylcholine (ACh) is released from the nerve into the synaptic cleft and binds to the nicotinic and muscarinic ACh receptors on the post-synaptic membrane relaying the signal from the nerve. For the body to perform any action, neurons have to be sent to the location in the body that the action will take place. When these neurons reach the presynaptic nerve, ACh goes and binds to its receptors, which release the secondary messages to initiate the given action. Once the secondary messages have been relayed, ACh has completed its part in relaying the action signal. ACh must then be removed, and that action is completed by AChE. The enzyme AChE is responsible for breaking ACh down into acetate and choline molecules. The choline molecules are recycled and reused to make more ACh to put in storage for future actions. In summary, ACh molecules are used by the body to initiate action signals.[6]

However, if AChE's function is inhibited, the ACh molecules will accumulate and cause an increase of action signals. The enhanced stimulation caused by this accumulation will cause the body to continue the action that is being signaled without ceasing. This increase in signal can have both negative and positive effects. Cholinesterase inhibitors, due to their effect in the signaling pathway, can be used as both toxic agents and aids for specific diseases. These inhibitors are also used to help prevent against chemical warfare. Pope et al discuss several aspects of cholinesterase inhibitors to educate readers on their affect in the body and to provide insight into their potential.[7] These aspects discussed by Pope et al include the history of cholinesterase inhibitors and the development of the mechanism of action for these compounds. Other aspects include the pharmacology and toxicology of these inhibitors and their use as drugs for specific diseases and chemical warfare prevention. The final aspects provide examples of

many drugs and their specific mechanisms of action as cholinesterase inhibitors and research study examples that demonstrate the effectiveness of these inhibitors.[7]

Both reversible and irreversible AChE inhibitors exist. PYR is a reversible inhibitor. Reversible inhibitors will bind to AChE for a short period of time, causing AChE to lose its function, but will eventually undergo decarbamylation. The decarbamylation causes PYR or any other reversible inhibitor to be released from AChE. Once the reversible inhibitor has been removed, AChE regains its function and begins to break down the ACh that has built up in the synapse. However, there are irreversible inhibitors. One example of irreversible inhibitors is organophosphorous compounds, which can be used as nerve agents. These compounds bind to AChE and permanently inhibit its function. When AChE completely loses its ability to break down ACh, those secondary receptors become overstimulated causing involuntary movements, excessive secretions, and respiratory dysfunction. Exposure to these compounds can cause acute toxicity, death, or other severe long-term effects like psycho-motor functioning impairment.[6]

Rang et al discuss cholinergic transmission in their book on pharmacology.[8] Since PYR is an AChE inhibitor, it affects the cholinergic transmission process. As mentioned before, AChE is an enzyme responsible for breaking down ACh after it has been used to help signal an action. This chapter discusses this process as well as what happens when an inhibitor is introduced. This chapter is helpful because it provides the pharmacology foundation necessary for an understanding of the action of this drug.[8]

2.2.2 PYR as a Treatment for Myasthenia Gravis

As an AChE inhibitor, PYR affects the nervous system. Ropper et al wrote a chapter on myasthenia gravis, which is a neuromuscular junction disorder that is treated by PYR. Myasthenia gravis translates to mean “grave muscular weakness.” This chapter by Ropper et al discusses PYR’s uses for treatment as well as the history of the disease.[9] Also, the medical

encyclopedia on MedlinePlus has a section discussing myasthenia gravis. As mentioned previously, PYR is a drug used to help treat people with this disorder. This source provides the background information on the disorder, which is helpful for understanding the full impact of this drug on the people who take it.[10]

The first use for PYR was for the treatment of myasthenia gravis. This disease causes fluctuating weakness of the voluntary muscle groups, which are all of the muscle groups that can be controlled. The fluctuating weakness occurs because a person with myasthenia gravis produces antibodies that target the ACh receptors and cause them to degrade. The degradation of receptors results in a loss of sites for ACh to bind. When the body sends neurons to designate a specific action after a loss of receptor sites, ACh will have difficulty finding somewhere to bind to continue the signaling pathway for that action to happen. A decrease in signal causes the muscle weakness. When a patient takes PYR, the resulting accumulation of ACh will cause the signaling to reach almost normal levels. The increased number of neurotransmitters in the synapse compensates for the loss of receptor sites.[11]

A supplement to an article by Zha et al in *Neurology* features the abstract for a study that used PYR to help treat patients that had been exposed to botulinum toxin.[12] The abstract contains the study's objective, background, method, results, and conclusion. Since PYR has been shown to be effective in patients with myasthenia gravis, the hope for this study was that PYR could be used to help treat the long-term effects of botulin toxicity. Currently, the abstract is the only part of this article that can be found. The goal is to retrieve the entire article as soon as it is released to read their full discussion on the treatment, use, and effect of PYR in this study.[12]

2.2.3 PYR as a Protection Against Nerve Agents

Shortly after PYR's use for myasthenia gravis was discovered, the Gulf War began. With the war came worry and fear of nerve agents being used in combat. Since PYR is a reversible

AChE inhibitor, scientists discovered that it could be used to bind to AChE to prevent the irreversible binding of nerve agents. PYR acts as a shield against nerve agents. These organophosphorous compounds, as discussed before, bind to AChE and permanently inhibit its function. However, if the nerve agents arrive and PYR is already bound to the AChE enzyme, then the nerve agents will not be able to bind. By blocking the enzyme against nerve agents, PYR protects AChE from permanent inactivation.[13] As the Gulf War began and the threat of nerve agents spread, PYR for the soldiers was given to the commanding officers in 21 pill packets. Each pill was 30 mg, to be taken three times a day. The commanding officer was responsible for determining whether the soldiers took the medicine. If the officer thought there was a threat for nerve agent attack, then the soldiers would be commanded to begin taking the regimental dose.[14] PYR, however, was not the soldier's only defense against nerve agents. On their person, each soldier carried atropine and was instructed to self-inject it into the bloodstream upon any encounter with a nerve agent. So these two drugs in combination were the defense against nerve agent intoxication.[13]

After the conclusion of the Gulf War, a study conducted by Abou-Donai et al looked at the effects of taking PYR.[15] This study focused on three different chemicals, one being PYR, to see if there could potentially be lasting effects from exposure to people using these chemicals for their respective protections. Abou-Donai et al used animal models to observe the effects over a 60-day period. No animal died after taking or being exposed to any of the individual three chemicals. However, animals taking any combination of these chemicals have mortality rates ranging from 20 to 80 percent. The study provides valuable information about PYR's use as a nerve gas agent and the potential long-term effects that soldiers could face from taking PYR for their protection.[15]

2.2.4 PYR as an Aid for Cardiac Function

The new use for PYR is as an aid for cardiac function. A study by Durand et al used pyridostigmine on mice during an experience of cardiac arrest.[16] The researchers induced a heart attack in each mouse and then administered PYR as a treatment in the following days and weeks. Researchers observed that this drug was able to preserve the cardiac autonomic balance, which is a positive result. This study provides hope that PYR could be used on human patients who have weaker hearts or who have suffered heart attacks. The patient plasma samples received were from patients taking the drug to help with cardiac function. This article provides insight as to how and why this drug could help.[16]

2.2.5 PYR Studies

Several studies have been conducted to analyze for PYR. In an article by Zhao et al, studies from 1975 to 2005 were discussed and compared that were for detecting PYR and its metabolites in biological samples. These biological samples included plasma and urine from rats and humans, plasma from guinea pigs, and serum from humans. The extraction methods used in these studies were either solid phase extraction or liquid-liquid extraction. Detection assays used within these studies were HPLC, ultraviolet (UV) techniques, gas chromatography, and mass spectrometry. All human plasma studies detected PYR using UV techniques.[17] No studies were mentioned that detected PYR using LC-MS/MS instrumentation.

The guinea pig study mentioned in the Zhao et al article was conducted by Needham et al and publish in the Journal of Chromatograph B in 2003. This study used LC-MS/MS instrumentation to detect for PYR in guinea pig plasma. The plasma samples in this study were prepared using a protein precipitation extraction with acetonitrile. Linearity, accuracy, precision, specificity, selectivity, stability, extraction recovery, and matrix effects studies were performed

for the method used in this study. Each of these method studies was successful, which means that the method used to analyze for PYR is acceptable and trustworthy.[5]

2.3 Laboratory Techniques

To help prove that PYR has made it into the bloodstream to aid in cardiac function, an extraction and LC-MS/MS method needed to be developed. A study conducted by Abu-Qare and Abou-Donai developed a method to detect Pyridostigmine and other drugs in rat plasma and urine samples. The researchers used a liquid-liquid extraction to separate the drugs from the plasma and urine. They analyzed these extracted samples using a high performance liquid chromatography (HPLC) instrument. This study can be a helpful reference as the PYR LC-MS/MS method for analysis is developed.[18]

2.3.1 LC-MS/MS

An LC-MS/MS method provides a way to separate and detect specific compounds. The LC portion separates compounds in a mixture so that they are easier to analyze and detect. The MS instrument is set to detect specific mass-to-charge ratios that accompany compounds; so when the compounds leave the LC column and hit the MS, the computer will know exactly which compound is being detected. The *Principles of Forensic Toxicology* provides several chapters that discuss how the LC and MS function. The chromatography chapter provides useful information on several key steps in the LC process. These key steps include mobile phase solution, chemical separation, and instrument detection information.[19] The chapter on mass spectrometry provides information on all of the processes that occur within the MS instrument during chemical analysis.[20] Having an understanding of the instrumentation being used is important for any researcher. These chapters will be a great resource to solidify current knowledge on how these instruments work.

2.3.2 Solid Phase Extraction (SPE)

An extraction method provides the mechanism for separating a drug from the specimen matrix in which it arrives. For this project, the drug is PYR and the matrix is plasma. The LC-MS/MS instruments are sensitive and expensive. If pure plasma samples were injected into these instruments, the tubing would clog and have to be replaced after each run. As a means to save time and money, extraction methods separate the desired drug from all other parts of the specimen. The extraction method used for this project will be a SPE. The books *Principles of Forensic Toxicology* and *Forensic Chemistry* provide insight into the SPE process. Siek in *Principles of Forensic Toxicology* provides a short section in his specimen preparation chapter on SPE. In this section, Siek discusses the advantages for using SPE. He also provides the steps necessary to successfully complete a SPE experiment.[21] Bell in *Forensic Chemistry* provides an in-depth analysis on the SPE column and the way that this column works to separate chemicals. Bell's chapter has a detailed figure on SPE procedures and a well-organized table on solvent strength, which are both key pieces of information to know before developing any SPE method.[22] These chapters will be a guide for building the SPE method.

2.4 Conclusion

Development of an extraction and LC-MS/MS method for the detection of PYR was needed to help prove the effectiveness and efficiency of PYR with cardiac patients. Originally, PYR was developed and used to help treat myasthenia gravis by increasing muscle function. Due to PYR's ability to reversibly inhibit AChE, it was then used as prevention for nerve agent attacks during the Gulf War. Now, PYR is being tried as a treatment to help improve cardiac function in patients with weak hearts. The development of this detection method is a necessary step in the process of providing evidence that PYR is useful as an aid for cardiac patients.

CHAPTER III

METHODOLOGY

3.1 Introduction

The experimental methodology for this study was developed to analyze pyridostigmine bromide (PYR) in plasma. This method consists of an extraction followed by liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis. Adherence to the Oklahoma State University Institutional Review Board rules were met by filing a form to request for the determination of non-human subject research because this study involved de-identified human specimen. The purpose of this study was to develop an LC-MS/MS method to accurately detect and quantify the amount of PYR in cardiac patient plasma samples.

3.2 Materials

Materials used for this method included the reagents, drug standards, other chemicals, plasma and laboratory equipment. Methanol from J.T. Baker Chemicals (Avantor Performance Materials, Center Valley, PA), acetonitrile from EMD Millipore (Darmstadt, Germany), and

formic acid from EMD Chemicals (Gibbstown, NJ) were all HPLC grade reagents. The PYR standard was from USP (Rockville, MD), and the internal standard (neostigmine bromide) was from Sigma-Aldrich (St. Louis, MO). Other chemicals used in this method include ammonium formate from Alfa Aesar (Ward Hill, MA), citric acid from VWR International (West Chester, PA), and dibasic anhydrous sodium phosphate from AMRESCO (Solon, OH). Pooled normal human plasma was obtained from Innovative Research (Novi, MI).

The laboratory equipment used for this study included a VWR B2500A-MTH sonicator, an Eppendorf Centrifuge 5424, a SPEware Cerex 48 Positive Pressure SPE, and a SPEware Cerex 48 Sample Concentrator. Both SPEware instruments were connected to a nitrogen gas cylinder. Other materials used are Oasis WCX 3cc, 60mg, 30µm cartridges from Waters, 0.45µm SFCA Nalgene syringe filters from Thermo Scientific, 15mL centrifuge tubes with flat caps (falcon tubes) from VWR, 13 x 100 culture tubes, 1.5mL centrifuge tubes, 1.5mL autosampler vials with caps, and 150µL vial inserts.

3.3 Reagent Preparation

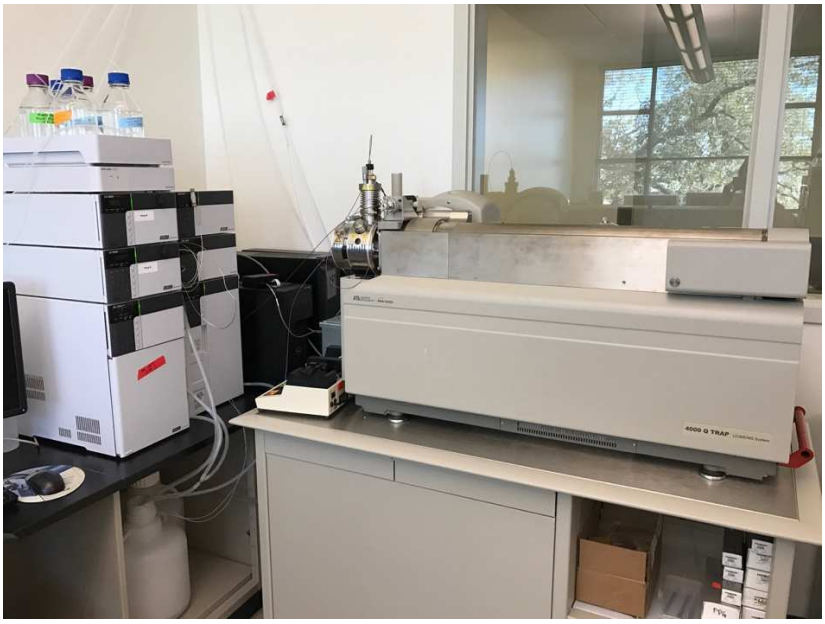
The reagents needed for the sample preparation include a 50mM citrate phosphate buffer solution and a citrate phosphate internal standard solution. The citrate phosphate buffer solution is made by combining 60.6mL of a 0.2M dibasic sodium phosphate solution with 39.4mL of a 0.1M citric acid solution. This combined solution is then diluted with 100mL of deionized water to make a 50mM citrate phosphate buffer solution. For the internal standard solution, 2.5µL of a 1 mg/mL neostigmine bromide stock solution made in a 1% formic acid in methanol solution is added to 24.997 5 mL of the 50mM citrate phosphate buffer solution. A 1 mg/mL PYR solution made in a 1% formic acid in methanol solution is used to make the 5000 ng/mL PYR stock solution that preps the calibration curve.

Mobile phase A (MPA) and mobile phase B (MPB) solutions are need for the LC instrument and the sample diluent. MPA is 1000mL of a 50mM ammonium formate solution at a pH of 3.25 in deionized water. MPB is a mixture of 500mL acetonitrile and 500mL methanol. The sample diluent is made by adding 3mL of MPA to 7mL of MPB. An elution buffer to pull PYR off of the solid phase extraction (SPE) column is made by combining 80mL of methanol and 20mL of a 1.5% formic acid solution in water.

3.4 Instrumentation

The instrumentation for this project included a Shimadzu HPLC system with a SCIEX 4000 Q-Trap mass spectrometry unit. Analyst® software and MultiQuant® software were used to control the instruments and perform data analysis. The column used in the LC system was a Restek Ultra PFPP. The entire LC-MS/MS instrument can be seen in Figure 1.

Figure 1. LC-MS/MS Instrumentation



This figure shows the entire LC-MS/MS instrument. The liquid chromatography instrument is on the far left of the picture and has the mobile phase solutions sitting on top. The mass spectrometry instrument is located on the right side of the picture. Both instruments are connected with tubing that allows the injected solutions to flow from the LC to the MS.

3.5 Extraction

An extraction is necessary for this method to separate PYR from any other chemicals that could be in the plasma matrix that it comes in. The extraction portion of this method also cleans up the sample that is injected onto the LC-MS/MS instrument. A clean sample keeps the tubing and column from clogging too quickly, which saves both time and money.

3.5.1 Calibration Curve Preparation

Pooled normal human plasma or blank plasma is used to prepare the standard curve samples in the calibration curve. The blank plasma arrives in a 100mL container, which is divided into 10mL portions in labeled falcon tubes. The blank plasma is stored in the freezer, so it must be set out to thaw at room temperature for about an hour before the process can begin. After the plasma has thawed, the plasma samples in the falcon tubes are placed in the sonicator for 20 minutes to separate the plasma from the proteins and clotting factors that could clog the extraction column. The sonicated plasma samples are then transferred to eppendorf tubes each containing approximately 1mL of plasma and then centrifuged to separate any leftover components from the plasma. The left behind clotting factors and proteins from the plasma were then discarded. Afterward, these plasma samples are filtered into culture tubes and ready to be used to prepare the calibration curve standards.

The standard curve is prepared by following the instructions from Table 1. Concentrations in the standard curve range from 1 to 50 ng/mL with quality control samples at concentrations of 5, 15, and 40 ng/mL. The 50 ng/mL standard is prepared first using the 5000 ng/mL PYR stock solution. This standard is made by combining 21µL of the 5000 ng/mL PYR stock solution with 2079µL of the blank plasma into a labeled centrifuge tube, which results in a total volume of 2100µL. Then 1400µL of this solution is used to make the 40 ng/mL, which leaves a remaining volume of 700µL for the 50 ng/mL standard. The rest of the standard solutions

are made following this serial dilution pattern. Two sets of calibration curves are prepared following these instructions: one for the calibration curve and the other for the quality control samples.

Table 1. Calibration Curve and Quality Control Sample Preparation Chart

Calibration Standard (ng/mL)	Solution Used	Spike Volume (µL)	Plasma Volume (µL)	Final Volume (µL)	Remaining Volume (µL)
50	5000	21	2079	2100	700
*40	50	1400	350	1750	700
25	40	1050	630	1680	720
*15	25	960	640	1600	700
10	15	900	450	1350	755
*5	10	595	595	1190	700
2.5	5	490	490	980	700
1	2.5	280	420	700	700
*QC		**Total**	5654		

This table represents the formula for how to prepare the standard curve and qualitative control samples. The far left column represents the range of concentrations included in the calibration curve. The asterisks represent the quality control samples.

While the curve is being prepared, patient samples should be removed from the -80 °C freezer to thaw for approximately one hour. Once the curve is prepared and the patient samples have thawed, the standards and patient samples are mixed with the internal standard solution. Adding internal standard into the individual samples allows for the quantification or

determination of the exact concentration of PYR in each sample. The internal standard and sample mixture is made by adding 500µL of the citrate phosphate internal standard solution to 500µL of the standard solution, quality control sample, or patient plasma sample in 1.5mL centrifuge tubes that are labeled with identifiable information. Blank and double blank samples are included in the batch to check for carryover of PYR from the standard samples. The blank was made by mixing 500µL of the citrate phosphate internal standard solution and 500µL of blank plasma. The double blank was made by mixing 500µL of citrate phosphate buffer and 500µL of blank plasma.

3.5.2 SPE Instructions

The WCX cartridge columns are set in a stand above a waste collection dish and labeled with the corresponding names of samples that will be eluted through them. These columns are conditioned using methanol and the 50mM citrate phosphate buffer solution. To begin this process, 2 mL of methanol is added to each column. Once the methanol has completely eluted through each column, 2 mL of citrate phosphate buffer is added to each column. After these two solutions have gone through the columns, the standard curve, quality control, and patient samples are slowly inserted into their designated columns. These samples are eluted at one drop per second using the positive pressure manifold until each sample has cleared the column. To rid the column of any compound other than PYR and the internal standard, the columns are then washed with 1 mL of the citrate phosphate buffer followed by 1 mL of methanol. After the methanol wash is complete, the columns are dried under maximum pressure for 30 minutes by the positive pressure manifold.

Culture tubes are positioned below the SPE columns for the elution of PYR and the internal standard. One mL of the elution buffer is added to each column and pushed through with the positive pressure manifold at approximately one drop per second. The elution buffer is used to

pull PYR and the internal standard from the SPE column to be analyzed. These eluent samples are then placed on the sample concentrator at 20 psi for about one hour or until they are completely dry. Then, 100 μL of sample diluent is added to each of the culture tubes and vortexed for 10 seconds to reconstitute the drug standards and patient samples. These reconstituted samples are placed into 150 μL inserts inside 1.5 mL autosampler vials to be put onto the LC-MS/MS instrument for analyzation.

3.6 LC-MS/MS Method Development

After the extraction phase has been completed, the final product needs to be analyzed to confirm the presence and the amount of analyte in the sample. The LC-MS/MS instrument provides a way to separate and analyze chemicals in a mixture. The LC portion of the instrument uses mobile phases that flow through the instrument's tubing and column to carry the sample through the instrument. The LC column has an interior surface that has affinities for specific compounds. The differences in affinities between the compounds and column help to separate samples with multiple compounds. The MS portion of the instrument is set to detect specific mass-to-charge ratios for the compounds and ions that come off of the LC column at their respective times of elution. The MS provides a way to monitor which drugs elute through the column at what time. These results are displayed on a chromatograph.

3.6.1 LC Parameters and Conditions

The conditions used for the LC include a binary flow pumping mode with a total flow of 0.5 mL/min that is set at a 70% Pump B concentration, which means that 70% of the total flow will be from the MPB solution. The oven temperature was set for 40 °C and a maximum temperature of 90 °C. The settings for the autosampler are shown in Table 2, and the time program for the instrument is displayed in Table 3.

Table 2. LC Autosampler Settings

Rinsing Volume	200 µL
Needle Stroke	52 mm
Rinsing Speed	35 µL/sec
Sampling Speed	15.0 µL/sec
Purge Time	25.0 min
Rinse Dip Time	3 sec
Rinse Mode	Before and after aspiration
Cooler Temperature	15 °C
Control Vial Needle Stroke	52 mm

This table shows all of the autosampler settings used for the LC-MS/MS method.

Table 3. Time Program for the LC-MS/MS Method

	Time	Module	Event	Parameter
1	6.10	Pumps	Pump B Conc.	70
2	6.10	Pumps	Total Flow	0.5
3	6.11	Pumps	Pump B Conc.	5
4	6.11	Pumps	Total Flow	0.7
5	7.10	Pumps	Pump B Conc.	5
6	7.11	Pumps	Pump B Conc.	95
7	8.10	Pumps	Pump B Conc.	95
8	8.11	Pumps	Pump B Conc.	70
9	8.11	Pumps	Total Flow	0.7
10	8.20	Pumps	Total Flow	0.5
11	9.00	Controller	Stop	

This table represents the time program used for the LC-MS/MS method. The time column represents the exact time in minutes that a specific event will occur whether that is a change in Pump B concentration or a change in the total flow rate.

3.6.2 MS Parameters and Conditions

The MS conditions are a scan type of multiple reaction monitoring (MRM) with a positive polarity. The total scan time is 0.42 seconds, and the total method lasts for 9.003 minutes. For this MS method, there are 1286 cycles, and each cycle is 0.42 seconds. Table 4 shows the MRM data for both the parent and daughter ions of PYR and neostigmine. This table also shows the MS parameters necessary for each ion.

Table 4. MRM Data for PYR and Neostigmine (NEO)

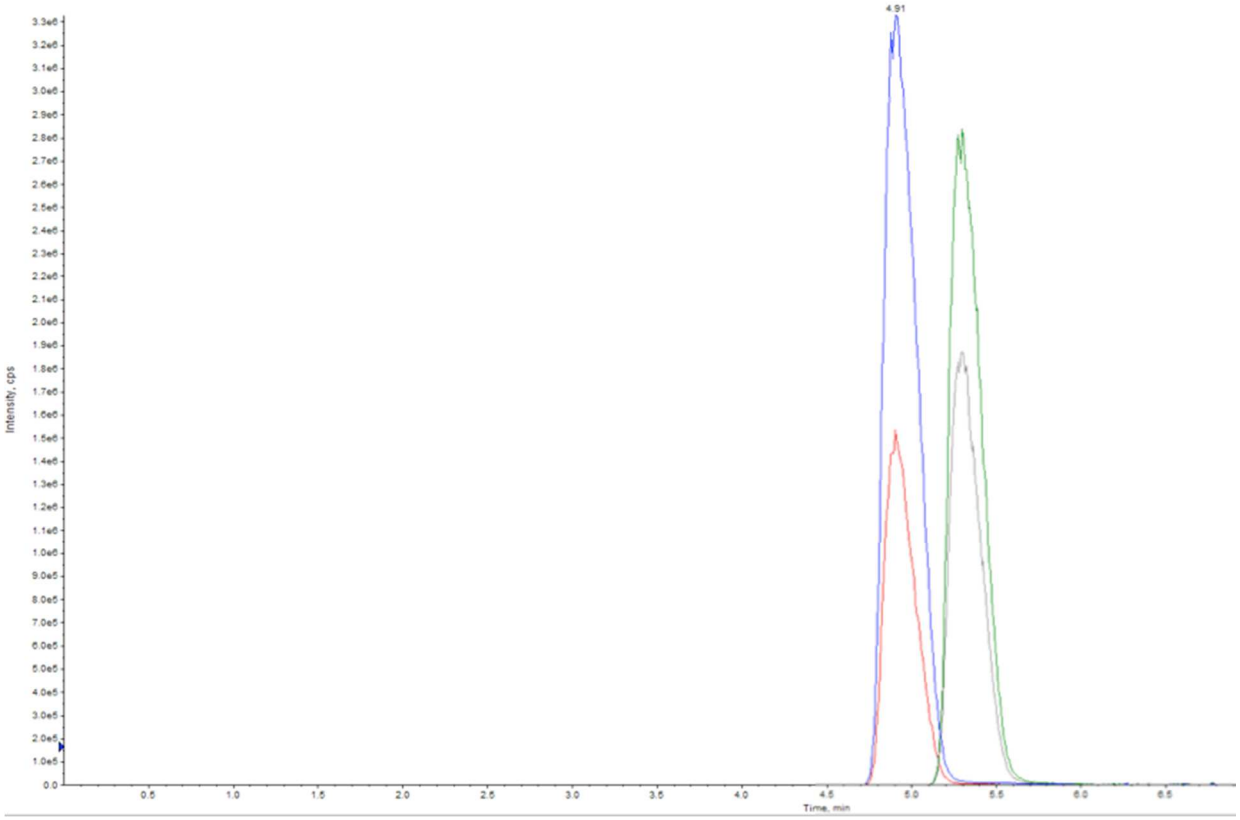
	ID	Q1 Mass (Da)	Q3 Mass (Da)	Time (msec)	DP (Volts)	CE (Volts)	CXP (Volts)
1	PYR	182.214	71.700	100.0	46.000	35.000	10.000
2	PYR	182.214	125.100	100.0	46.000	25.000	18.000
3	NEO	223.200	150.000	100.0	40.000	49.000	0.000
4	NEO	223.200	56.000	100.0	40.000	83.000	8.000

The data represented in this table are the MRM and MS settings for this method. The parent ions for PYR and NEO are the mass-to-charge ratios listed in the Q1 column, and their daughter ions are the ones shown in the Q3 column. The DP, CE, and CXP columns represent the MS settings necessary to detect each ion.

3.7 Data Analysis

The data collected from the LC-MS/MS method include the peaks for every compound injected, which are the calibration curve standards, the quality control samples, and the patient plasma samples. The chromatogram peaks were integrated for each sample. Also, the resulting concentrations for each sample were calculated by the software and then recorded in an Excel log. Figure 2 represents an example of a chromatograph for the 50 ng/mL standard. The MultiQuant® software was used to integrate and quantify each sample.

Figure 2. Chromatogram of the 50 ng/mL Standard



This figure displays a chromatogram for the 50 ng/mL standard in the calibration curve. The vertical axis is the intensity of the peak, and the horizontal axis is the time in minutes. Each of the two peaks represents a different compound. The blue peak is the PYR peak, and the red peak within it is a qualifier ion used to confirm that the compound is PYR. The green peak is the internal standard peak, and the grey peak within it is also a qualifier ion. The retention times for PYR and the internal standard are 4.9 and 5.3 minutes, respectively.

3.8 Validation

Validation for this experimental methodology followed the guidelines made by the Scientific Working Group for Forensic Toxicology (SWGTOX). The validation for this method included studies on linearity, accuracy, precision, matrix effects, process efficiency, recovery efficiency, interference, carryover, and stability. Results for each of these studies fell within the required ranges.

3.9 Conclusion

The experimental methodology for this study was to conduct an extraction for PYR in plasma and then perform LC-MS/MS analysis on the resulting samples. Blank plasma was properly prepared for use to make the calibration curve standards and qualitative control samples. All samples were added to an internal standard solution, so that the exact concentration of each sample could be calculated. Each sample was then extracted by SPE to separate PYR and the internal standard from the undesired chemicals in the plasma matrix. The extracted samples were then injected into the LC/MS instrument to determine the concentrations of the calibration curve, qualitative control, and patient samples. Analyst® and MultiQuant® were used to control the instruments and perform data analysis.

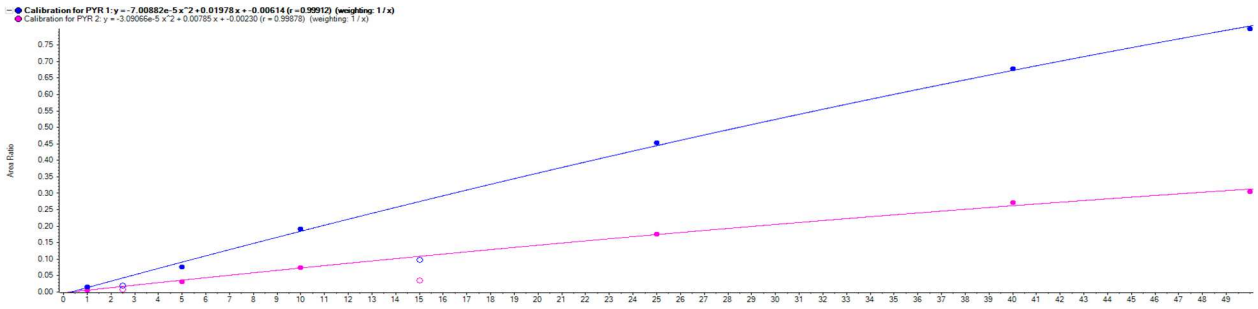
CHAPTER IV

FINDINGS

4.1 Calibration Curve

The project began with testing and validating the calibration curve. Figure 3 displays the first attempt at the calibration curve. This curve produced two outliers. The second attempt at the curve also produced outliers. Figure 4 shows the second attempt at the calibration curve.

Figure 3. Calibration Curve from 1/20/2016

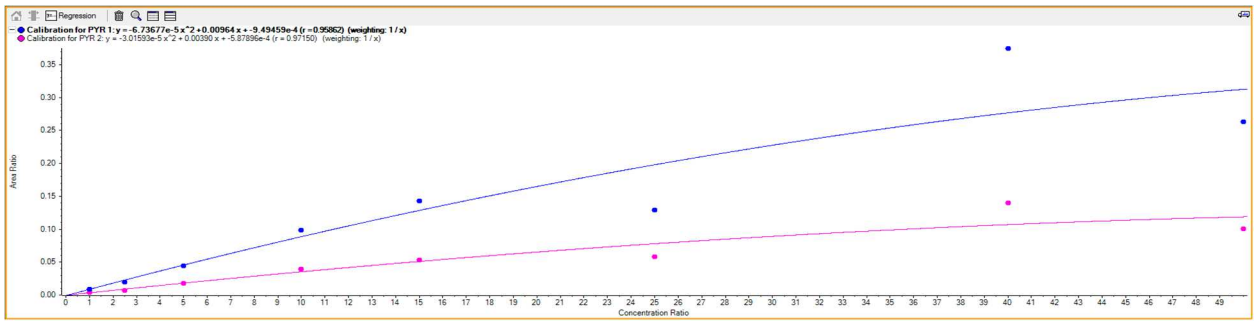


This calibration curve was the first attempt at the process. The standard values 2.5 and 15 ng/mL (the points that are not filled in) were removed from the curve because they were outliers. The y-axis represents the area ratio between the standard and the internal standard, and the x-axis represents the concentration ratio between the standard and the internal standard.

With these random outliers appearing in different standard positions, a change to the plasma

preparation was made, which is the preparation mentioned in the method section. The original preparation only thawed the blank plasma before preparing the calibration curve, which left proteins and clotting factors that clogged the SPE columns not allowing all the drug to elute through.

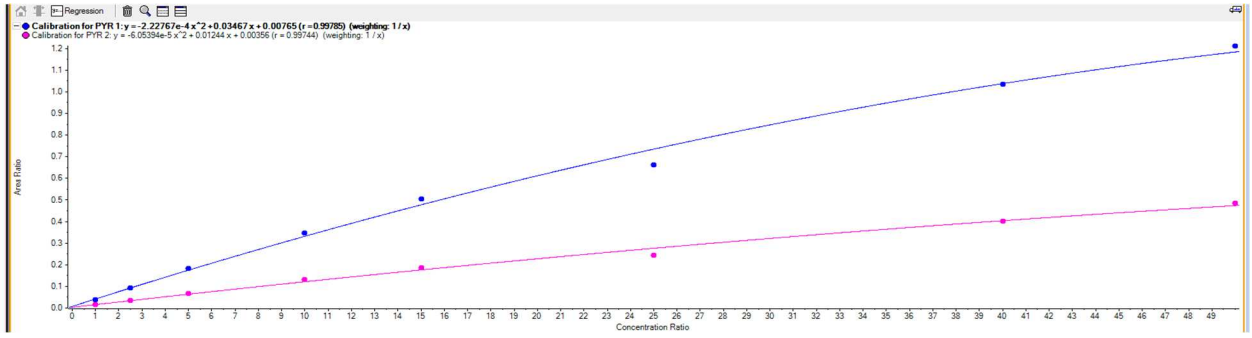
Figure 4. Calibration Curve from 2/1/2016



The second attempted calibration curve produced at least two outliers: the 25 and 40 ng/mL samples.

The calibration curves after the plasma preparation method change produced better linearity, which meant that the calibration curve could be validated. Figures 5, 6, 7 display the reproducible calibration curves that led to the validation of the method.

Figure 5. Calibration Curve from 2/17/2016



This calibration curve was the first attempt after implementing the new plasma preparation method. Every point in the curve is within 15% of the expected concentration.

Figure 6. Calibration Curve from 3/1/2016

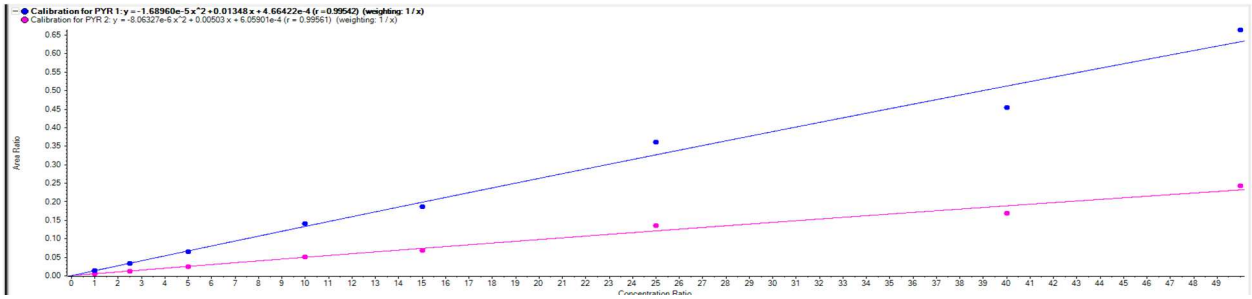


Figure 6 represents the first successful replication of the calibration curve after the plasma preparation method implementation. Again, every point is within 15% of the expected concentration and there are no distinct outliers.

Figure 7. Calibration Curve from 3/22/2016

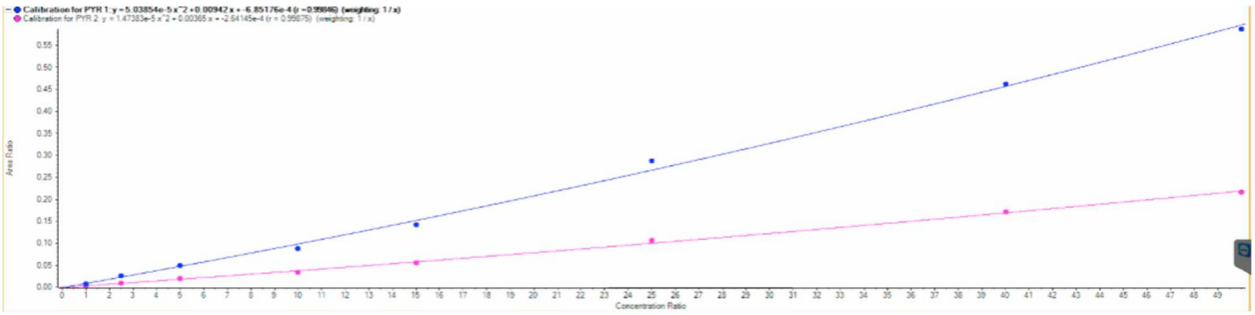


Figure 7 represents the final successful replication of the calibration curve before the validation began. Once again, each point is within 15% of the expected concentration.

4.2 Validation Results

Next the calibration curve and LC-MS/MS method were validated according to SWGTOX guidelines, and the data and results for each validation phase are reported below.

4.2.1 Linearity

Table 5 represents the data taken for the seven calibration curves tested for linearity.

Table 5. Linearity Data for Calibration Curve Validation

Concentration (ng/mL)	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6	Run 7	Average	Absolute Error (%)
50	55.3	52.03	55.00	53.25	56.18	53.99	52.03	53.97	7.94
40	40.4	41.39	39.67	39.67	38.96	40.88	40.71	40.23	0.58
25	24.7	23.43	24.86	24.78	24.57	25.08	25.52	24.70	1.20
15	15.6	15.56	15.08	15.22	15.50	15.68	15.32	15.41	2.73
10	10.1	10.23	9.92	10.13	10.26	10.48	10.26	10.20	2.00
5	5.14	4.97	5.34	5.33	5.29	5.10	5.19	5.20	4.00
2.5	2.59	2.56	2.65	2.60	2.59	2.64	2.55	2.60	4.00
1	0.90	0.92	0.91	0.89	0.92	0.95	0.99	0.93	7.00
Blank*	0	0	0	0	0	0	0	0	
R^2	0.995	0.998	0.995	0.998	0.991	0.998	1.000	0.996	

This table displays the data from the linearity study for the calibration curve. The far left column lists the expected concentration value, and the columns below Runs 1-7 list the actual values from the individual runs. The R^2 value for each run is above 0.99. The percent absolute error for each standard point is less than 10%. The results from this study validate the calibration curve's linearity.

4.2.2 Accuracy

The accuracy studies were completed using the values of the quality control (QC) standards from five different run days. Each QC standard was run at least four times each day with the calibration curve and patient samples. Tables 6-8 provide the accuracy data for each QC standard. The overall accuracy for all of the QCs was 101%.

Table 6. Accuracy Data for 40 ng/mL Quality Control Standard

Day #	Result 1	Result 2	Result 3	Result 4	Daily Mean	Daily Mean Accuracy (%)
1	42.3	41.0	34.9	38.3	39.1	98%
2	37.6	38.0	38.3	38.1	38.0	95%
3	37.0	43.8	41.7	41.1	40.9	102%
4	39.4	36.9	36.4	37.7	37.6	94%
5	42.9	36.0	41.1	38.3	39.6	99%
					Average	98%

Table 6 displays the data for QC 1, which is 40 ng/mL pyridostigmine. The average accuracy percentage for all runs was 98%, and the range of values for accuracy % were 94-102%.

Table 7. Accuracy Data for 15 ng/mL Quality Control Standard

Day #	Result 1	Result 2	Result 3	Result 4	Daily Mean	Daily Mean Accuracy (%)
1	16.2	15.7	14.3	15.1	15.3	102%
2	14.6	14.4	14.7	14.6	14.6	97%
3	13.9	15.4	15.8	15.3	15.1	101%
4	15.0	14.9	14.5	15.2	14.9	99%
5	15.8	14.2	15.6	14.8	15.1	101%
					Average	100%

Table 7 displays the data for QC 2, which is 15 ng/mL pyridostigmine. The average accuracy percentage for all five runs was 100%, and the range of accuracy percentage values was 97-102%.

Table 8. Accuracy Data for 5 ng/mL Quality Control Standard

Day #	Result 1	Result 2	Result 3	Result 4	Daily Mean	Daily Mean Accuracy (%)
1	5.1	5.7	5.1	5.2	5.3	106%
2	5.1	5.2	5.0	5.1	5.1	102%
3	5.2	5.5	5.6	5.5	5.5	109%
4	5.5	5.5	5.4	5.5	5.5	109%
5	5.6	5.1	5.4	5.3	5.3	107%
					Average	106%

Table 8 represents the data for QC 3, which is 5 ng/mL pyridostigmine. The average accuracy percentage for all five runs was 106%, which is within 10% of the expected concentration. The range of accuracy percentages was 102-109%, so no day had a mean accuracy percentage more than 10% away from the desired result.

4.2.3 Precision

The data used for the precision studies were from the same set of QC standards used for the accuracy study. Table 9 shows the results for within-run, between run, within-laboratory, interday, and intraday precision for each QC standard.

Table 9. Precision Data for All Quality Control Standards

Imprecision	**40 ng/mL**	**15 ng/mL**	**5 ng/mL**
Within-Run Precision	6.20	4.03	4.22
Between Run Precision	4.16	3.20	2.18
Within-Laboratory Precision	6.22	4.11	4.49
Interday Precision	1.45	1.22	2.56
Intraday Precision	1.38	1.46	2.04

Table 9 displays the precision results for all QC standards. Each value for precision is beneath 15, which is the acceptable limit.

4.2.4 Matrix Effects, Recovery Efficiency, and Process Efficiency

The data listed in Table 12 are from the individual runs for the neat, post-extraction, and pre-extraction samples. The data from these three different extraction types were used to determine matrix effects (ME), recovery efficiency (RE), and process efficiency (PE). The neat samples are pyridostigmine samples at 25 ng/mL made in methanol and injected directly onto the instrument without extraction. The post-extraction samples are blank plasma samples are reconstituted with the 25 ng/mL methanol standard after the full extraction process, and the pre-extraction samples are 25 ng/mL samples made following the actual method procedure. Table 13 lists the results from each study.

Table 10. ME, RE, and PE Data

	Neat Area	Post Area	Pre Area
Sample1	2.4E+06	2.8E+06	2.4E+06
Sample2	2.5E+06	2.6E+06	2.6E+06
Sample3	2.4E+06	2.5E+06	2.5E+06
	Neat Area	**Post Area**	**Pre Area**
Average	2.4E+06	2.7E+06	2.5E+06

Table 12 shows the data from each extraction. The areas for each of the three samples for the neat, post-extraction, and pre-extraction runs are listed, and the averages for each are given at the bottom of the table.

Table 11. Results from ME, RE, and PE Study

Matrix Effect (%)	Recovery Efficiency (%)	Process Efficiency (%)
110	93.8	103

Table 13 shows the results for each of the three studies. The matrix effect percentage was calculated by dividing the post-extraction average area by the neat average area and then multiplying by 100. The recovery efficiency percentage was calculated by dividing the pre-extraction average area by the post-extraction average area and then multiplying by 100. Finally, the process efficiency percentage was calculated by dividing the pre-extraction average area by the neat average area and then multiplying by 100. All values are roughly within 10% of 100%, which is better than the values that are needed for validity.

4.2.5 Stability

The stability study tests a 40 ng/mL sample's stability on the instrument following three days. Table 14 provides the data for each injection on the instrument for each day.

Table 12. Stability Study Data

Date	Sample ID	Area	Ratio of PYR to NEO	Absolute Error (%)
7/28/16	PYR 1	3.05E+07	0.970	0
	PYR 2	1.29E+07	0.638	0
	NEO 1	3.15E+07		
	NEO 2	2.02E+07		
7/29/16	PYR 1	4.50E+07	0.974	0.47
	PYR 2	1.85E+07	0.607	4.84
	NEO 1	4.62E+07		
	NEO 2	3.04E+07		
7/30/16	PYR 1	4.24E+07	0.997	2.83
	PYR 2	1.72E+07	0.634	0.56
	NEO 1	4.25E+07		
	NEO 2	2.72E+07		

Table 14 provides the data for the stability study. PYR and NEO stand for pyridostigmine and neostigmine, and the 1 and 2 represent the two daughter ions detected by the mass spectrometer. The ratios for PYR 1 to NEO 1 and PYR 2 to NEO 2 were determined for each day. The absolute error percentage was calculated for 7/29 and 7/30 comparing them to the 7/28 run. All percentages are within 5% of the first day's ratios, which means that the on-instrument stability for this extracted drug is acceptable.

4.2.6 Carryover

The carryover study was done to make sure that pyridostigmine was not staying on the column between runs, which could cause the next calculated concentration to be higher than in actuality. Carryover was tested by injecting the 50 ng/mL standard and then a blank sample to make sure that no drug was detected in the blank. The data for this study is shown in Table 15.

Table 13. Carryover Study Data

Concentration	Sample	Area
50	PYR 1	1.40E+07
50	PYR 2	5.54E+06
50	NEO 1	3.47E+07
50	NEO 2	2.22E+07
Blank	PYR 1	N/A
Blank	PYR 2	N/A
Blank	NEO 1	3.40E+07
Blank	NEO 2	2.21E+07
50	PYR 1	2.34E+07
50	PYR 2	9.59E+06
50	NEO 1	3.27E+07
50	NEO 2	2.07E+07
Blank	PYR 1	N/A
Blank	PYR 2	N/A
Blank	NEO 1	3.34E+07
Blank	NEO 2	2.10E+07
50	PYR 1	2.18E+07
50	PYR 2	9.08E+06
50	NEO 1	3.26E+07
50	NEO 2	2.11E+07
Blank	PYR 1	N/A
Blank	PYR 2	N/A
Blank	NEO 1	3.21E+07
Blank	NEO 2	2.12E+07

Table 15 provides the data for the carryover study. Pyridostigmine was not detectable in any blank run, which means that there is no carryover of this drug with this method and column.

4.2.7 Interference

The final validity study conducted was the interference study. This study compared an extracted 40 ng/mL sample of pyridostigmine with an extracted 40 ng/mL sample that had fifty-seven other drugs added. This interference study was conducted to ensure that the patient concentrations of pyridostigmine would not be influenced by other drugs that the patient could be taking. Table 16 provides the interference study data.

Table 14. Interference Study Data

Concentration (ng/mL)	Sample ID	Area	Ratio of PYR to NEO	Error (%)
40	PYR 1	3.25E+07	0.816	
40	PYR 2	1.33E+07	0.518	
40	NEO 1	3.98E+07		
40	NEO 2	2.58E+07		
40 (Interference)	PYR 1	2.40E+07	0.788	-3.46
40 (Interference)	PYR 2	9.54E+06	0.494	-4.55
40 (Interference)	NEO 1	3.05E+07		
40 (Interference)	NEO 2	1.93E+07		

Table 16 provides the data for the interference study. The absolute error percentages for the interference sample were each less than 5%. Also, each ratio decreased by about the same percentage, which drug interference should not be a problem when calculating pyridostigmine's concentration.

4.3 Patient Data

The calibration curves, Figures 8-14, and patient results, Tables 17-23, are provided for each day that patient samples were extracted and quantified.

Figure 8. Calibration Curve for Patient Samples Ran on 4/6/2016

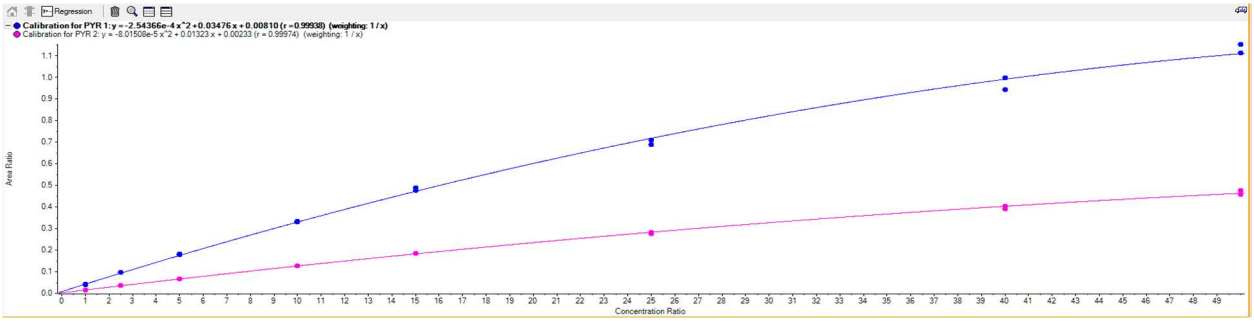


Figure 8 provides the graph for the calibration curve that accompanied patients 51 and 008. All standards and QCs fell within 15% of their expected values.

Table 15. Patient Data for 4/6/2016

Patient	ng/mL	Patient	ng/mL
51-1 V2	N/A	008-01 V2	N/A
51-1 V3	N/A	008-01 V3	N/A
51-2 V3	7.67	008-02 V3	8.01
51-1 V4	N/A	008-01 V4	11.4
51-2 V4	N/A	008-02 V4	8.59
51-1 V5	9.90	008-01 V5	1.93
51-2 V5	9.79	008-02 V5	3.29
51-3 V5	16.2	008-03 V5	5.83
51-1 V6	N/A	008-01 V6	26.6
51-2 V6	N/A	008-02 V6	17.7
51-1 V7	N/A	008-01 V7	10.7
51-2 V7	3.29	008-02 V7	19.0
51-3 V7	27.7	008-03 V7	40.4
51-1 V8	N/A	008-01 V8	24.5
51-2 V8	N/A	008-02 V8	21.4
51-1 V9	N/A		
51-1 V10	N/A		
51-2 V10	N/A		
51-1 V11	N/A		
51-2 V11	N/A		
51-1 V12	N/A		

Table 17 provides the concentration of pyridostigmine in each of the plasma samples for patients 51 and 008. The V followed by a number indicates the visit number, and the 01, 02, and 03 indicate the order that the samples were taken from the patient on that visit. The value N/A indicates that no pyridostigmine was detected in that sample.

Figure 9. Calibration Curve for Patient Samples Ran on 4/12/2016

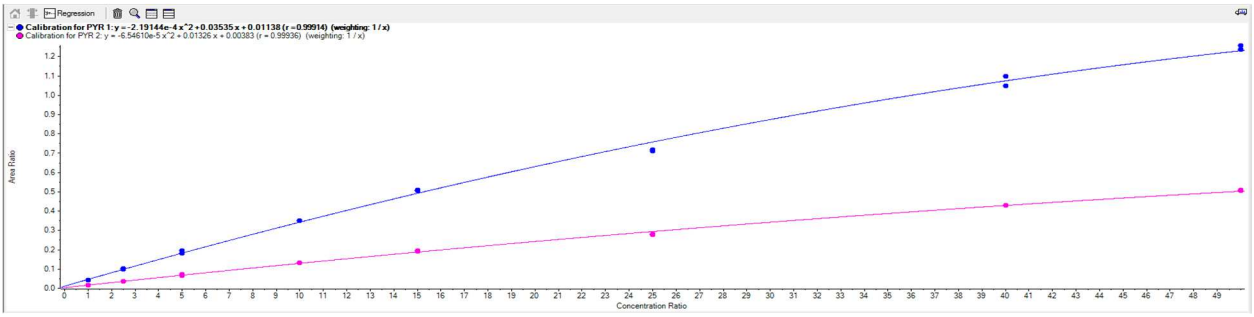


Figure 9 provides the graph for the calibration curve that accompanied patients 49 and 007. All standards and QCs fell within 15% of their expected values.

Table 16. Patient Data for 4/12/2016

Patient	ng/mL	Patient	ng/mL
49-1 V2	N/A	007-01 V2	N/A
49-1 V3	N/A	007-01 V3	N/A
49-2 V3	3.01	007-02 V3	4.42
49-1 V4	12.7	007-01 V4	4.50
49-2 V4	12.1	007-02 V4	8.87
49-1 V5	12.3	007-01 V5	10.6
49-2 V5	13.7	007-02 V5	9.59
49-3 V5	16.7	007-03 V5	20.2
49-1 V6	17.8	007-01 V6	13.3
49-2 V6	17.9	007-02 V6	18.5
49-1 V7	19.3	007-01 V7	14.1
49-2 V7	17.9	007-02 V7	15.4
49-3 V7	29.0	007-03 V7	28.0
49-1 V8*	Not Quantifiable	007-01 V8	39.4
49-2 V8*	Not Quantifiable	007-02 V8	28.5
49-1 V9*	Not Quantifiable		
49-1 V10	24.9		
49-2 V10	19.7		
49-1 V11	24.0		
49-2 V11	21.3		
49-1 V12	N/A		

Table 18 provides the concentration of pyridostigmine in each of the plasma samples for patients 49 and 007. The V followed by a number indicates the visit number, and the 01, 02, and 03 indicate the order that the samples were taken from the patient on that visit. The value N/A indicates that no pyridostigmine was detected in that sample. Samples with asterisks were unable to be quantified with pyridostigmine peaks present, so they were diluted and rerun in a later batch.

Figure 10. Calibration Curve for Patient Samples Ran on 4/15/2016

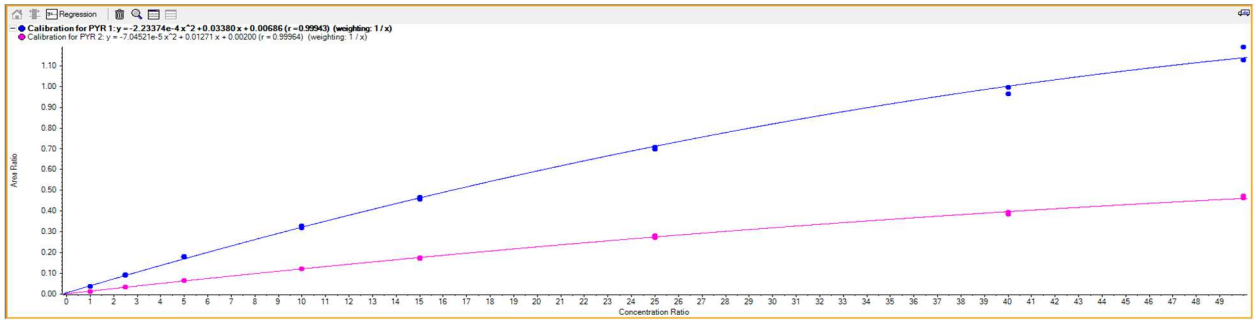


Figure 10 provides the graph for the calibration curve that accompanied patients 33 and 24. All standards and QCs fell within 15% of their expected values.

Table 17. Patient Data for 4/15/2016

Patient	ng/mL	Patient	ng/mL
33-1 V2	N/A	24-1 V2	N/A
33-1 V3	N/A	24-1 V3	N/A
33-2 V3	9.38	24-2 V3	6.62
33-1 V4	5.94	24-1 V4	11.3
33-2 V4	5.00	24-2 V4	9.56
33-1 V5	8.04	24-1 V5	6.32
33-2 V5	7.00	24-2 V5	7.46
33-3 V5	8.43	24-3 V5	10.2
33-1 V6	12.9	24-1 V6	14.2
33-2 V6	19.9	24-2 V6	10.4
33-3 V6	20.3	24-1 V7	8.41
33-1 V7	15.7	24-2 V7	6.46
33-2 V7	12.2	24-3 V7	11.9
33-3 V7	25.5	24-1 V8	33.8
33-1 V8	26.1	24-2 V8	28.8
33-2 V8	20.1	24-1 V9	31.1
33-1 V9	23.7	24-1 V10	8.18
33-1 V10	10.1	24-2 V10	12.4
33-2 V10	8.20	24-1 V11	4.33
33-1 V11	12.4	24-2 V11	8.27
33-2 V11	10.2	24-1 V12	N/A
33-1 V12	N/A		

Table 19 provides the concentration of pyridostigmine in each of the plasma samples for patients 33 and 24. The V followed by a number indicates the visit number, and the 01, 02, and 03 indicate the order that the samples were taken from the patient on that visit. The value N/A indicates that no pyridostigmine was detected in that sample.

Figure 11. Calibration Curve for Patient Samples Ran on 4/23/2016

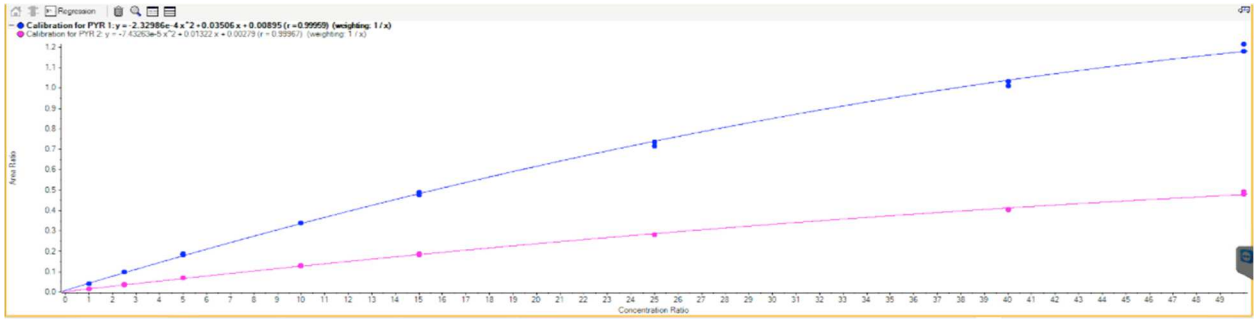


Figure 11 provides the graph for the calibration curve that accompanied patients 44 and 40. All standards and QCs fell within 15% of their expected values.

Table 18. Patient Data for 4/23/2016

Patient	ng/mL	Patient	ng/mL
44-1 V2	N/A	40-1 V2	N/A
44-1 V3	N/A	40-2 V3	3.90
44-2 V3	10.6	40-1 V4	11.6
44-1 V4	7.65	40-2 V4	10.7
44-2 V4	6.83	40-1 V5	6.65
44-1 V5	N/A	40-2 V5	17.3
44-2 V5	2.25	40-3 V5	18.9
44-3 V5	14.4	40-1 V6	19.0
44-1 V6	25.8	40-2 V6	13.5
44-2 V6	23.0	40-1 V7	11.6
44-1 V7	25.4	40-2 V7	29.0
44-2 V7	22.3	40-3 V7	24.4
44-3 V7	34.6	40-1 V8	14.8
44-1 V8	25.4	40-2 V8	13.2
44-2 V8	23.0	40-1 V9	23.7
44-1 V9	33.0	40-1 V10	17.2
44-2 V9	27.6	40-2 V10	15.1
44-1 V10	19.6	40-1 V11	7.43
44-2 V10	22.9	40-2 V11	5.64
44-1 V11	13.3	40-1 V12	N/A
44-2 V11	9.43		
44-1 V12	N/A		

Table 20 provides the concentration of pyridostigmine in each of the plasma samples for patients 44 and 40. The V followed by a number indicates the visit number, and the 01, 02, and 03 indicate the order that the samples were taken from the patient on that visit. The value N/A indicates that no pyridostigmine was detected in that sample.

Figure 12. Calibration Curve for Patient Samples Ran on 5/11/2016

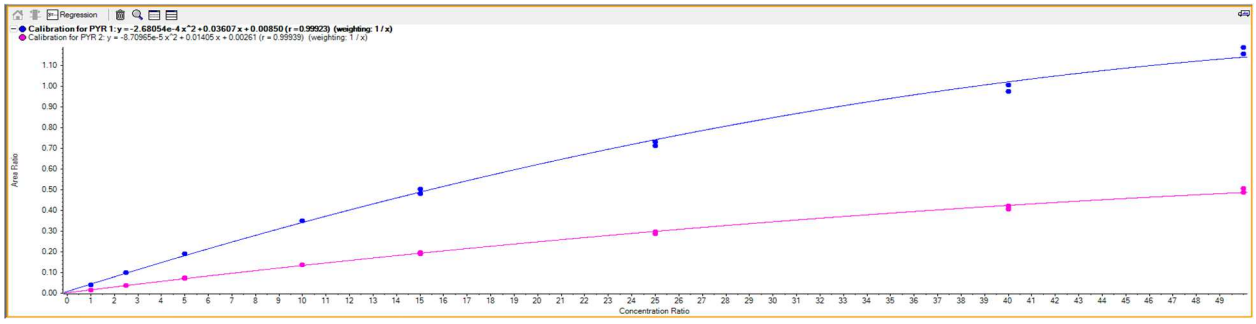


Figure 12 provides the graph for the calibration curve that accompanied patients 35, 31, and 12. All standards and QCs fell within 15% of their expected values.

Table 19. Patient Data for 5/11/2016

Patient	ng/mL	Patient	ng/mL	Patient	ng/mL
35-1 V2	N/A	31-1 V2	N/A	12-1 V2	N/A
35-2 V3	7.21	31-1 V3	N/A	12-1 V3	N/A
35-1 V4	14.4	31-2 V3	15.7	12-2 V3	7.54
35-2 V4	12.6	31-1 V4	1.41	12-1 V4	16.5
35-1 V5	9.21	31-2 V4	4.52	12-2 V4	14.6
35-2 V5	7.02	31-1 V5	11.8	12-1 V5	12.0
35-3 V5	8.63	31-2 V5	18.8	12-2 V5	7.94
35-1 V6	17.0	31-3 V5	19.9	12-3 V5	9.30
35-2 V6	15.6	31-1 V6	12.2	12-1 V6	10.8
35-1 V7	20.9	31-2 V6	5.35	12-2 V6	9.39
35-2 V7	19.9	31-1 V7	13.6	12-1 V7	3.35
35-3 V7	29.3	31-2 V7	11.6	12-2 V7	7.38
35-1 V8	22.7	31-3 V7	47.4	12-3 V7	7.38
35-2 V8	21.2	31-1 V8	12.3	12-1 V8	8.00
35-1 V9	15.2	31-2 V8	9.28	12-2 V8	5.98
35-2 V9	15.0	31-1 V9	17.1	12-1 V9	4.51
35-1 V10	16.7	31-1 V10	7.09	12-1 V12	N/A
35-2 V10	21.4	31-2 V10	6.94		
35-1 V11	12.1	31-1 V12	N/A		
35-2 V11	12.0				
35-1 V12	N/A				

Table 21 provides the concentration of pyridostigmine in each of the plasma samples for patients 35, 31, and 12. The V followed by a number indicates the visit number, and the 01, 02, and 03 indicate the order that the samples were taken from the patient on that visit. The value N/A indicates that no pyridostigmine was detected in that sample.

Figure 13. Calibration Curve for Patient Samples Ran on 5/15/2016

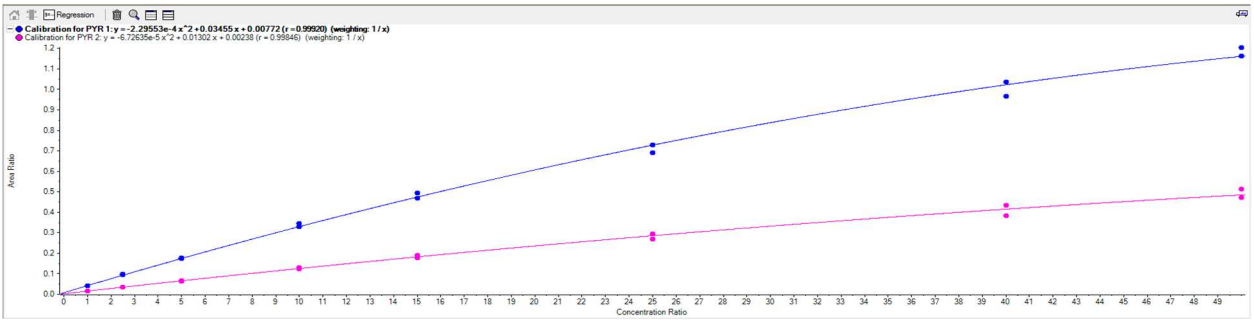


Figure 13 provides the graph for the calibration curve that accompanied patients 009, 23, 001, 11, and 28. All standards and QCs fell within 15% of their expected values.

Table 20. Patient Data for 5/15/2016

Patient	ng/mL	Patient	ng/mL	Patient	ng/mL
009-1 V2	N/A	11-1 V3	N/A	28-1 V2	N/A
009-1 V3	N/A	11-2 V3	8.50	28-1 V3	N/A
009-2 V3	9.99	11-1 V4	25.6	28-2 V3	7.92
009-1 V8	19.6	11-2 V4	26.7	28-1 V4	16.7
009-2 V8	15.9	11-1 V5	17.6	28-2 V4	17.4
009-1 V9	30.7	11-2 V5	16.8	28-1 V5	5.58
009-1 V10	11.0	11-3 V5	33.8	28-2 V5	5.62
009-2 V10	8.65	11-1 V6*	Not Quantifiable	28-3 V5	25.4
009-1 V11	N/A	11-2 V6*	Not Quantifiable	28-1 V6*	Not Quantifiable
23-2 V2	N/A	11-1 V7*	Not Quantifiable	28-2 V6	38.1
23-1 V5	1.15	11-2 V7*	Not Quantifiable	28-1 V7	25.6
23-2 V5	7.35	11-3 V7*	Not Quantifiable	28-2 V7	34.5
23-3 V5	15.2	11-1 V8*	Not Quantifiable	28-3 V7*	Not Quantifiable
23-1 V6	10.5	11-2 V8*	Not Quantifiable	28-1 V9	N/A
23-2 V6	8.20	11-1 V9*	Not Quantifiable	28-1 V10	19.1
001-1 V3	N/A	11-1 V10	32.9	28-2 V10	14.6
001-2 V3	4.88	11-2 V10	35.3	28-1 V11	2.47
001-1 V4	13.5	11-1 V11	23.4	28-2 V11	1.78
001-2 V4	12.5	11-2 V11	27.9	28-1 V12	N/A
001-1 V5	9.03	11-1 V12	N/A		
001-1 V9	N/A				

Table 22 provides the concentration of pyridostigmine in each of the plasma samples for patients 009, 23, 001, 11, and 28. The V followed by a number indicates the visit number, and the 01, 02, and 03 indicate the order that the samples were taken from the patient on that visit. The value N/A indicates that no pyridostigmine was detected in that sample. Samples with asterisks were unable to be quantified with pyridostigmine peaks present, so they were diluted and rerun in the next batch.

Figure 14. Calibration Curve for Diluted Patient Samples Ran on 5/27/2016

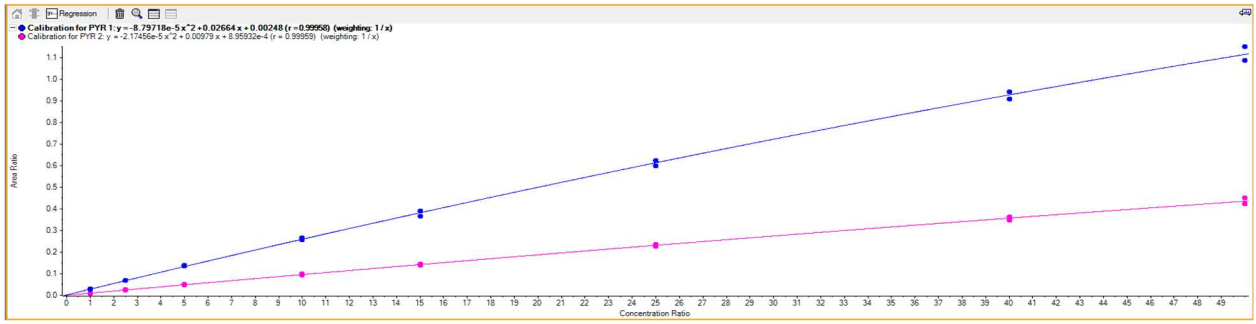


Figure 14 provides the graph for the calibration curve that accompanied the samples that needed to be diluted. All standards and QCs fell within 15% of their expected values.

Table 21. Patient Data Following Dilution on 5/27/2016

Patient	ng/mL	Diluted
49-1 V8	88.1	8.81
49-2 V8	67.2	6.72
49-1 V9	82.5	8.25
28-1 V6	32.1	3.21
28-3 V7	47.0	4.70
11-1 V6	9.34	0.93
11-2 V6	10.1	1.01
11-1 V7	14.2	1.42
11-2 V7	39.1	3.91
11-3 V7	82.9	8.29
11-1 V8	13.4	1.34
11-2 V8	7.46	0.75
11-1 V9	13.5	1.35

Table 23 provides the dilution information for the patients whose samples were unable to be quantified in earlier runs. All samples were diluted by a factor of 10, so the concentrations under the Diluted column are those quantified by the software. The values beneath the ng/mL column are the actual concentrations after the dilution is taken out.

CHAPTER V

CONCLUSION

5.1 Discussion

PYR has already proved that it is a useful AChE inhibitor by helping with myasthenia gravis patients and soldiers during the Gulf War that were threatened with nerve agent attacks. Recently, it has also proven its effectiveness as a cardiac aid. The results from this research provide quantitative data from patients taking specific doses of this drug. This data provides the means to determine a proper dosing regimen so that this drug can be taken by and provided to patients correctly.

This study also provides a method for treating blank plasma before SPE. The original method was to thaw and then use the thawed plasma to prepare the calibration curve. However, the large proteins and clotting factors within the plasma clogged the SPE columns, which inhibited all of PYR from eluting through the column. The clogged columns led to poor and inconsistent calibration curves with low intensities for each standard. However, when the method was altered to thawing, sonicating, centrifuging, and then filtering the plasma, cleaner plasma was produced that kept the columns for clogging. This new method then allowed for the calibration curve to be validated.

5.2 Conclusion

The LC-MS/MS method was successful in detecting and quantifying PYR in the cardiac patient plasma samples. The plasma preparation and SPE methods were able to provide clean samples to be analyzed on the LC-MS/MS instrumentation. The results from this study provide the quantified PYR sample data needed to help determine proper dosing for future clinical patients. This study also provides a successful extraction and LC-MS/MS method that could be incorporated into any future lab that may need to quantify PYR in human plasma.

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